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Identification of SSR Markers Associated with Resistance to Nematode *Tylenchorhynchus brevilineatus* Infection in Groundnut (*Arachis hypogaea* L.)

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ABSTRACT

Kalahasti malady is a disease caused by the soil-borne nematode, *Tylenchorhynchus brevilineatus* that occurs during the post-rainy season in Andhra Pradesh, India. Screening of segregating populations is very difficult as maintenance of a sick plot at the experimental farm is not possible. Hence, identification of molecular markers is a research priority. Among 35 SSR markers used for the study, the alleles of two primers (i.e., 225, 260, 275, 310 and 330 base pair (bp) alleles of pPGS seq14H6 and 210 bp allele of pPGS seq 16G8) showed an association with resistance to Kalahasti malady when seven parents (Tirupati 3, Kalahasti, Narayani, Kadiri 6, Tirupati 4, Prasuna and ICG (FDRS) 79), six F_1s and 25 genotypes were screened. Confirmation of the association between resistance and markers has to be further carried out in mapping populations i.e. F_2 populations and recombinant inbred lines, which are now being developed.

Keywords: alleles, groundnut, Kalahasti malady, nematode, SSR

INTRODUCTION

Groundnut is an important oil seed crop of India and in the state of Andhra Pradesh. In India, the crop is cultivated on 6.2 million ha with a production of 7.3 million tonnes (mt) in two major climacteric situations i.e. 80% in rain-fed and 20% in *rabi* i.e., summer. In Andhra Pradesh, it is grown on 1.8 million ha with a production of 1.6 mt. In about 0.3 million hectares (m ha), it is cultivated during *rabi* with a productivity of 1932 kg/ha (Annual Report of Directorate of Groundnut Research 2009). Out of this, coastal sands along the sea coast and river belts of different rivers in Chittoor, Nellore, Kadapa and Prakasam districts constitute approximately 0.05 m ha.

A nematode disease of groundnut (*Arachis hypogaea* L.), termed 'Kalahasti malady', is a serious concern in the coastal sands. Among the plant parasitic nematodes isolated from the soil in the rhizosphere region of the affected groundnut crop, the nematode *Tylenchorhynchus brevilineatus* Williams was more abundant than others (Reddy *et al*. 1984; Sharma 1988; Prasad and Rangappa 1994; Naidu 1996). It was also found to be associated with the roots of sugarcane (*Saccharum officinarum* L.) and castor (*Ricinus communis* L.) in Haryana and Bihar States (Gupta 1986). It caused a significant reduction in plant growth in wheat (*Triticum aestivum* L.) var. 'Sonalika' wherein yellowing, stunting, reduced tillering with reduced grain yields were also reported (Thakar *et al.* 1986).

As a result of nematode infection, affected groundnut plants appeared stunted with greener-than-normal foliage (Reddy *et al*. 1984). The disease appears initially as small yellow to brown lesions on roots, pegs, pod stalks and young developing pods. As the disease progresses, the lesions turn brown with cracked margins. These lesions later turn from deep brown to black that coalesce and cover the whole pod surface (**Fig. 1**). The affected pods appear scabby after drying. In severe cases, a reduction in pod stalk length, pod size and kernel size also occur. Kernels from diseased pods apparently appear healthy. When the pegs are affected in initial stages, they fail to develop into pods, leading to severe yield losses. However, no symptoms on foliage (Ratna Kumar 1988; Naidu 1996), or differences in plant height were reported in diseased plants (Ratna Kumar 1988). Yield losses of 60-70% were reported in endemic areas (Rao *et al.* 1986).

Presently, the disease is managed through the application of chemical insecticides. Soil application of carbofuran 3G or aldicarb 10G granules at 4-6 kg a.i/ha not only reduced the nematode population in the soil and the intensity of disease symptoms on the pods but also significantly enhanced pod yield (Rao *et al*. 1986). However, this method was found to be too expensive for wide-spread adoption by farmers. Hence, there is a need to develop alternate methods to control the disease. Groundnut germplasm was screened against nematode infection to identify accessions or cultivars that were resistant or tolerant to nematode infection. A total of 48 genotypes of different habit groups were screened for their reaction to nematode infection. The test entries exhibited wide variation (16.22 to 89.91%) in their susceptibility to nematode infection. Among the genotypes screened, 'NC2' was resistant with < 20% infection. The genotypes 'NcAc17090' and 'TI-18-B_2' ('TCG 1706') recorded 30-40% and 61-70% infection, respectively while 71-80% infection was observed in 20 genotypes. Twenty five genotypes were highly susceptible with an 81-90% infection (Rao *et al*. 1986).

A total of 1599 groundnut germplasm accessions and breeding lines were screened in replicated trials on a farmer's field in a hot spot location during 1985-1986 by Mehan *et al.* (1993). 23 genotypes were identified as resistant and they had disease scores of 2.0 or < 2 on a 1-5 disease scale. Of these, 14 were confirmed to be resistant in advanced screening trials in the 1986 rainy and the 1985/6, 1986/7 post-rainy seasons. Resistance to the nematode (*T. brevilineatus*) was stable in all three trial sites in the 1986/7 post-rainy season. Most of the resistant genotypes had undesirable pod/seed characteristics. One of the resistant genotypes was a high-yielding breeding line 'TCG 1518'. It

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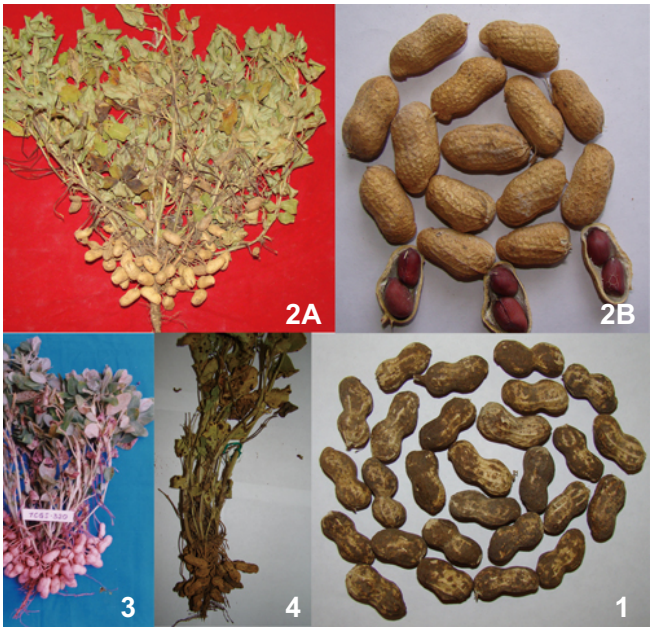

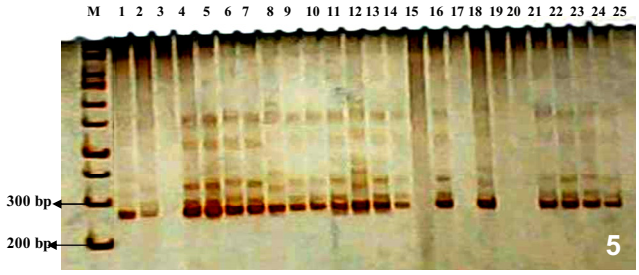

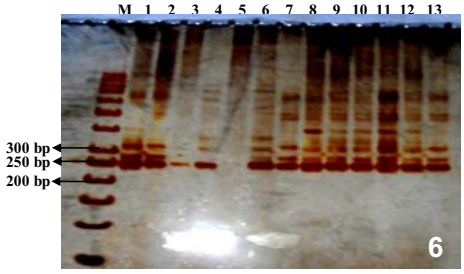

Fig. 1 Closer view of nematode affected pods in susceptible lines. **Fig. 2** (**A**) 'Tirupati 3', kalahasti malady resistant parent; (**B**) Healthy pods of 'Tirupati 3'. **Fig. 3** Resistant variety, 'Kalahasti'. **Fig. 4** Susceptible variety 'Tirupati 4'. **Fig. 5** Amplification products of primer pPGSseq14H6 in parents: M – 1Kb DNA ladder (Fermentas) 1. 'Ahbaya', 2. 'Tirupati 1', 3. 'ICGV 91114', 4. 'TLG 45', 5. 'TCGS 876', 6. 'ICGV 93468', 7. 'TCGS 584', 8. 'ICGV 99029', 9. 'JL 220', 10. 'JL 24', 11. 'K 134', 12. 'K 1375', 13. 'Tirupati 2', 14. 'TCGS 821', 15. 'Rohini', 16. 'ICGV 00350', 17. 'ICGV 07022', 18. 'ICGV 07217', 19. 'ICGV 06039', 20. 'ICGV 06138', 21. 'ICGV 87846', 22. 'TCGS 1043', 23. 'ICGV 03325', 24. 'ICGV 08021', 25. 'GPBD 4'. **Fig. 6** Amplification products of primer pPGSseq14H6 in parents and F1s: M - 50 bp DNA ladder (Fermentas), 1. 'Tirupati 3', 2. 'Kalahasti', 3. 'Narayani', 4. 'Tirupati 4', 5. 'Kadiri 6', 6. 'Prasuna', 7. 'ICG (FDRS)79', 8. 'Narayani' x 'Tirupati 3', 9. 'Tirupati 4' x 'Tirupati 3', 10. 'Kadiri 6' x 'Tirupati 3', 11. 'Tirupati 3' x 'ICG (FDRS 79)', 12. 'Prasuna' x 'Tirupati 3', 13. 'TCGS 320' x 'Tirupati 3'.

was subsequently released as 'Tirupati 3' for cultivation in nematode-infected areas in 1990 by the State Varietal Release Committee. 'Tirupati 3' is a Virginia bunch line that matures in 125-130 days. However, farmers require a variety with a shorter duration than 'Tirupati 3', i.e., one that matures in 105-110 days to fit into the cropping system of these areas.

In 1989, a hybridization programme was initiated involving 'Tirupati 3' (**Fig. 2A, 2B**) as the donor parent with high-yielding Spanish bunch lines with a shorter duration of maturation of 105-110 days. In the 1993/4 post rainy season, a total of 23 advanced breeding lines developed in the breeding programme were evaluated in a hot spot location on the Andhra Pradesh State Seed Development Corporation Farm, and Srikalahasti was screened for nematode infection on a 1-5 scale at the time of harvest: 1-2 resistant (1-25% damage), 2.1-3 moderately resistant (26-50%), 3.1 to 4 susceptible (51-75% damage) and 4.1 to 5 highly susceptible (more than 75%). Naidu and Moses (2004) screened 38 genotypes derived from the crosses, 'TCG 1716'×'TCG 1518', 'TCG 1717'×'TCG 1518' and 'TCG 273'×'TCG 1518' in a hot spot location at the Andhra Pradesh State Seed Corporation Farm at Srikalahasti in Chittoor district of Andhra Pradesh during the post rainy season of 1992-93 and 1993-94. Two check varieties, one resistant ('Tirupati 3') and one susceptible ('JL24') were included in the screening trial. None of the genotypes were immune to nematode infection. Three genotypes, 'TCGS 328' (41.7%), 'TCGS 353' (36.92%) and 'TCGS 357' (26.00%) and the resistant check variety, 'Tirupati 3' exhibited resistance to nematode attack with a score of 2.0. Total crude fibre content and total phenolic compounds in groundnut shells of resistant variety, 'Tirupati 3' were higher by 36.4 and 85.15%, respectively over the susceptible groundnut variety, 'JL24' (Naidu 1996). Three genotypes, 'TCGS 332' (43.50%), 'TCGS 337' (41.21%) and 'TCGS 341' (31.72%) – among moderately resistant genotypes – gave significantly higher pod yield over 'JL 24' (20.66 t/ha).

Two stabilized selections ('TCG 1709'×'TCG 1518')-54 and ('TCG 1709'×'TCG 1518')-8 were resistant to Kalahasti malady with a disease score of 2.0. These two selections matured in 110 days with a pod yield of 3.27 and 3.43 t ha^{-1}, respectively. The susceptible control variety, 'JL 24' recorded a disease score of 4.0 with a pod yield of 2.76 t ha^{-1}. Two selections ('TCG 1709'×'TCG 1518')-18 and ('TCG 1709'×'TCG 1518')-7, were moderately resistant with a pod yield of 4.00 and 3.94 t ha^{-1}, respectively (Vasanthi *et al.* 1998). In July 2002, one of these selections, 'TCGS 320' derived from 'TCG 1709'×'TCG 1518', was released as 'Kalahasti' (**Fig. 3**) by the Andhra Pradesh State Varietal Release Committee. Later on, a moderately susceptible line, 'TCGS 341', with a rose testa colour, was released as 'Prasuna' in 2006. To rectify the defects in these varieties and maintain high yield potential and resistance to Kalahasti malady, it is necessary to identify a robust screening tool. As Kalahasti malady is caused by soil-borne nematodes in specific types of soils in specific situations (e.g., sandy patches near river belts) the same type of situation cannot be created in experimental farms and screening has to be done in such hot spot locations. The appearance of Kalahasti malady is also uncertain depending on various climate factors, even in hot spot locations. Studies of Naidu (1996) on the mode of spread of the disease clearly established that it occurs only through soil infestation with nematode and not through affected pods and seeds from diseased pods. Due to variations in climatic and soil conditions it would be difficult to precisely identify resistant plants/progenies, even in hot spot locations.

In this context, the identification of molecular markers associated with nematode resistance is essential to aid in the development of high-yielding Kalahasti malady resistant variety with desirable quality traits (Mehan *et al.* 1997). Simple sequence repeats (SSRs), also known as microsatellites, are a class of molecular markers based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Lutty 1989). These repeat sequences are often highly polymorphic, even among closely related cultivars, due to slippage mutations during DNA replication causing variation in the number of repeating units. Different alleles of a given locus can be readily detected using primers designed from the conserved DNA sequences flanking the SSR and polymerase chain reaction (PCR). Microsatellite markers are analytically simple and readily transferable between different plant species (He *et al.* 2003). Studies of Song *et al.* (2010) indicated that SSR markers could detect more polymorphism in peanut than other molecular markers like RFLPs (Gimenes

et al. 2007), AFLPs (Gimenes *et al.* 2002) and RAPDs (Dwivedi *et al.* 2001). Naito *et al.* (2008) reported that SSR provided the most abundant information and the highest reproducibility among the three DNA fingerprinting techniques i.e., RAPD, AFLP and SSR from their study which involved 116 groundnut genotypes comprising 97 germplasm accessions of *A. hypogaea* L. originating from 9 countries and 19 wild species of genus *Arachis.* Angelici *et al.* (2008) employed microsatellite markers to distinguish the species and accessions of section Rhizomatosae, detecting high variation among accessions. Cuc *et al.* (2008) and Wang *et al.* (2007) found SSR markers to be informative and useful for breeding applications in crops like groundnut, which originated through a single and recent polyploidization event followed by successive selection during breeding efforts and hence have a limited genetic background.

The disadvantages of RAPD and AFLP markers were circumvented by the development of SSR markers because such markers are detected by long primers which have increased specificity and consequently, repeatability, allowing the evaluation of individual loci (Yu *et al.* 1999). Hoshino *et al.* (2006) found high transferability and polymorphism of heterologous microsatellite markers from the genus *Arachis*. Selvaraj *et al.* (2009) identified SSR markers linked to pod and kernel traits through bulk segregant analysis. Hopkins *et al.* (1999) reported a primer pair amplifying an (AT) 18 SSR located in the 3' untranslated region of the lectin gene. A total of 14 fragments were amplified and 10 out of the 20 groundnut accessions were differentiated.

Varma *et al*. (2005) used SSR markers to study intra- and inter-accession polymorphic variation among rust resistant and susceptible parents and to identify SSR markers associated with resistance to rust in two crosses of groundnut. In their study, 14 primers showed inter-accession polymorphic variation. Five SSR primer pairs were common between resistant and susceptible parents between two crosses 'ICGV 99005'×'TMV2' and 'ICGV 99003'× 'TMV2' but none of the markers of these five common SSRs were found to be associated with rust resistance; they concluded that the wild species *A. stenosperma* in the pedigree of resistant parents in 'ICGV 99003' and *A. batizocoii* in 'ICGV 99005' might be contributing different genes conferring resistance to rust.

The present study was undertaken to identify SSR markers associated with resistance to Kalahasti malady in a set of resistant and susceptible parents and to verify the association in a large number of genotypes with a known reaction to Kalahasti malady and F_1s involving resistant and susceptible parents.

MATERIALS AND METHODS

Plant materials

Seven parents, 'Tirupati 3' belonging to *Arachis hypogaea* subspecies *hypogaea* var *hypogaea*, 'Kalahasti', 'Narayani', 'Kadiri 6', 'Tirupati 4' (**Fig. 4**), 'Prasuna' and 'ICG (FDRS) 79' belonging to subspecies *fastigiata* variety *vulgaris* and six F_1s and 25 genotypes whose reaction to Kalahasti malady is known through earlier studies, constituted the experimental material (**Tables 2, 3**). The reaction of F_1s to Kalahasti malady was recorded by sowing them in a sick plot (in which Kalahasti malady regularly occurs naturally) during kharif, 2010 at the Regional Agricultural Research Station (RARS), Tirupati. Among the parents, 'Tirupati 3' (Mehan *et al.* 1993), 'ICG (FDRS) 79' (pers. obs.) and 'Kalahasti' (Vasanthi *et al.* 2003) are resistant to Kalahasti malady, 'Prasuna' is moderately resistant and 'Narayani', 'Kadiri 6' and 'Tirupati 4' are highly susceptible to Kalahasti malady (pers. obs.). 'Tirupati 3' is an isogenic selection with a solid red testa from 'TMV10', a variegated testa line belonging to ssp. *hypogaea* var. *hypogaea*. Kalahasti is a selection from 'TCG 1709'×'TCG 1518' belonging to sub-species *fastigiata* variety *vulgaris*. 'Prasuna' is derived from the cross 'TCG 1717'×'TCG 1518' belonging to sub-species *fastigiata* variety *vulgaris*. 'ICG (FDRS) 79' is a line obtained from ICRISAT which is derived from a double cross, ('ICGV 92069'×'ICGV 93184')×('ICGV 96246'×'92 R/75'). Susceptible lines, 'Narayani', 'Tirupati 4' and 'Kadiri 6' are all derived from 'JL 24'×'Ah613/S'.

Table 1 Annealing temperatures of SSR markers

Reference	Locus	Annealing temperature (^{O}C)	Nature of the marker
Ferguson *et al.* 2003	pPGS Seq 12F7	57	monomorphic
	pPGS Seq 17F6	58	monomorphic
	pPGP Seq 13A7	56	monomorphic
	pPGP Seq 2B10	57	monomorphic
	pPGS Seq 2F05	58	polymorphic
	pPGS Seq 14H6	58	polymorphic
	pPGP Seq 4G2	59	monomorphic
	pPGP Seq 4H11	60	monomorphic
	pPGP Seq 2D12B	60	polymorphic
	pPGS Seq 18C5	58	polymorphic
	pPGS Seq 16G8	59	polymorphic
	pPGP Seq 8D9	62	monomorphic
	pPGS Seq 10D4	63	polymorphic
	pPGP Seq 3D9	63	monomorphic
	pPGP Seq 3A8	63	polymorphic
	pPGP Seq 3A1	61	polymorphic
	pPGS Seq 15C10	64	monomorphic
	pPGP Seq 1B9	62	monomorphic
	pPGP Seq 7G2	65	monomorphic
	pPGS Seq 16C6	64	polymorphic
	pPGS Seq 13A10	55	no amplification
	pPGP Seq 5D5	60	polymorphic
	pPGP Seq 16F1	59	monomorphic
Cuc *et al*. 2008	IPAHM – 689	60	no amplification
	IPAHM – 103	59	monomorphic
	IPAHM – 165	60	monomorphic
	IPAHM – 105	58	no amplification
Hopkins *et al.* 1999	Ah – 041	60	polymorphic
	Ah – 193	60	no amplification
	Ah – 229	60	monomorphic
	Ah – 522	60	monomorphic
He *et al*. 2003	PM – 3	61	no amplification
	PM – 36	60	no amplification
	PM – 45	63	no amplification
Selvaraj *et al.* 2009	PM – 375	60	no amplification

DNA isolation

Total genomic DNA was isolated from fresh leaves of 20-25-day-old plants following a modified CTAB method (Saghai-Maroof *et al.* 1984; Doyle and Doyle 1987) as reported previously in Mace *et al.* (2003). The quality and quantity of DNA was determined using a Nanodrop spectrophotometer (ND-1000 UV/Vis, Thermo Scientific, USA).

SSR marker genotyping

35 SSR primer pairs (**Table 4**) (Eurofins Genomics India Pvt. Ltd, whitefield, Bangalore, India and Sigma Aldrich®, Mahadevapura, Bangalore, India) developed by Ferguson *et al.* (2003), Cuc *et al.* (2008), Hopkins *et al.* (1999) and He *et al*. (2003) were used for screening three resistant, one moderately resistant and three susceptible genotypes. PCR amplifications were performed in a 25-µl reaction volume, which consisted of 1X *Taq* buffer F (Tris pH9, KCl, TritonX-100 – 10X), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.6 µM of each forward and reverse primer, 0.4 µl of 3U/µl *Taq* DNA Polymerase (Genei™, Bangalore, India), 100-250 ng of genomic DNA, and 14.67 µl of sterile milli Q water (Millipore, Milli Q Gradient Century, Millipore in India, Bangalore, India). The concentrations of primer pairs were optimized individually. Samples were subjected to the following thermal profile for amplification (Eppendorf programmable thermal controller, Hamsburg, Germany). The profile was modified from that of Varma *et al.* (2005) with an initial denaturation step of DNA at 94°C for 2 min, followed by 35 cycles of amplification consisting of 94°C for 45 sec, 55-65°C for 1 min, and 72°C for 1 min 30 sec.

Table 2 SSR alleles of primer pair pPGS seq14H6 associated with resistance/susceptibility to Kalahasti malady in 32 genotypes and six F_1s.

Genotype / F_1	Reaction to Kalahasti malady	pPGS Seq 14H6 allele (bp) size
Tirupati 3	R	225, 260, 275, 310, 330
Kalahasti	R	225, 260, 275, 330
Narayani	S	240, 250
Tirupati 4	S	amplification absent
Kadiri 6	S	amplification absent
Ahbaya	S	250, 300, 350
TLG 45	MR	260, 275, 330
TCGS 876	MR	225, 260, 275, 330
ICG (FDRS) 79	R	225, 260, 275, 330
Tirupati 1	S	amplification absent
ICGV 91114	S	250
ICGV 93468	S	250
TCGS 584	MR	225, 260
ICGV 99029	S	250
JL 220	S	250
JL 24	S	250
K 134	S	amplification absent
K 1375	S	250
Tirupati 2	MR	250, 330
TCGS 821	S	250
Rohini	S	250
ICGV 00350	S	250
ICGV 07022	S	250
ICGV 07217	S	250
ICGV 06039	S	250
ICGV 06138	MR	225, 260, 275
ICGV 87846	S	amplification absent
TCGS 1043	S	240, 250
ICGV 03325	S	amplification absent
ICGV 08021	S	240, 250
GPBD 4	S	250
Prasuna	MR	225, 260, 275, 310, 330
Narayani X Tirupati 3 (F_1)	R	225, 260, 275, 310
Tirupati 4 X Tirupati 3(F_1)	R	225, 260, 275, 310, 330, 350
Kadiri 6 X Tirupati 3 (F_1)	R	225, 260, 275, 310, 330, 350
Tirupati 3 X ICG (FDRS) 79 (F_1)	R	225, 260, 275, 310, 330, 350
Prasuna X Tirupati 3 (F_1)	R	225, 260, 275, 310, 330
Kalahasti X Tirupati 3 (F_1)	R	225, 260, 275, 330

R- Resistant, S- Susceptible, MR- Moderately resistant

Table 3 SSR alleles of primer pair pPGS seq16G8 associated with resistance/susceptibility to Kalahasthi malady in 32 genotypes and six F_1s.

Genotype / F_1	Reaction to Kalahasti malady	pPGS Seq 16G8 allele (bp) size
Tirupati 3	R	210
Kalahasti	R	210
Narayani	S	amplification absent
Tirupati 4	S	amplification absent
Kadiri 6	S	amplification absent
Ahbaya	S	amplification absent
TLG 45	MR	210
TCGS 876	MR	210
ICG (FDRS) 79	R	210
Tirupati 1	S	amplification absent
ICGV 91114	S	amplification absent
ICGV 93468	S	amplification absent
TCGS 584	MR	210
ICGV 99029	S	190, 230
JL 220	S	230
JL 24	S	amplification absent
K 134	S	amplification absent
K 1375	S	amplification absent
Tirupati 2	MR	210
TCGS 821	S	amplification absent
Rohini	S	230, 240
ICGV 00350	S	amplification absent
ICGV 07022	S	amplification absent
ICGV 07217	S	amplification absent
ICGV 06039	S	amplification absent
ICGV 06138	MR	190, 210, 230
ICGV 87846	S	amplification absent
TCGS 1043	S	amplification absent
ICGV 03325	S	amplification absent
ICGV 08021	S	amplification absent
GPBD 4	S	230
Prasuna	S	amplification absent
Narayani X Tirupati 3 (F_1)	R	210
Tirupati 4 X Tirupati 3 (F_1)	R	210, 230
Kadiri 6 X Tirupati 3 (F_1)	R	210
Tirupati 3 X ICG (FDRS) 79 (F_1)	R	210
Prasuna X Tirupati 3(F_1)	R	210
Kalahasti X Tirupati 3 (F_1)	R	210

R- Resistant, S- Susceptible, MR- Moderately resistant

Annealing temperatures were optimized individually for each primer pair (**Table 1**). There was a final extension at 72°C for 10 min. The PCR products were separated on 9% non-denaturing PAGE gels and the products were subjected to silver staining using a modified Kolodny (1984) protocol. The stained gels were photographed to assess the DNA profiles. The primers that showed polymorphism in resistant and susceptible genotypes were used to further screen F_1s and 25 other genotypes other than parental genotypes used in the primary screening.

RESULTS AND DISCUSSION

Among the 35 SSR markers used for screening three resistant, one moderately resistant and three susceptible genotypes to Kalahasti malady, 10 SSR primers were found to be polymorphic. Two primer pairs, pPGS Seq 14H6 and pPGS Seq 16G8 showed distinct polymorphism in resistant and susceptible genotypes to Kalahasti malady.

These two primers pairs were employed to screen 32 genotypes (including seven parents) and hybrids belonging to six crosses involving parents with resistance and susceptibility to Kalahasti malady. The amplification profiles resulting from pPGS seq14H6 primer pair are shown in **Figs. 5** and **6**. This primer was highly polymorphic among the primers used in the present study. Jiang *et al.* (2007) reported amplification of eight alleles among 31 genotypes comprising 27 bacterial-resistant genotypes and four highly susceptible genotypes to bacterial wilt with this primer. This primer pair produced 10 amplification products i.e., alleles with sizes from 225 to 800 bp in the material used in the present study. Alleles 225, 260, 275, 310 and 330 bp in size were associated with Kalahasti malady resistance. The amplification products of pPGS seq16G8 primer pair were 190, 210, 230, and 240 bp. Among the alleles, the allele 210 bp in size was associated with resistance to Kalahasti malady.

Polymorphism in parents and hybrids

Among the parents screened, 'Tirupati 3' possesses absolute resistance (i.e., almost immune) to Kalahasti malady (Mehan *et al.* 1993). In this genotype, alleles of pPGS seq14H6 primer at 225, 260, 275, 310, 330, 400 and 475 bp were observed. The genotype 'Kalahasti' is a derivative of 'TCG 1709'×'Tirupati 3' and is resistant to Kalahasti malady with a score of 1-2 on the 1-5 disease scale (Vasanthi *et al*. 1998). In this genotype, except for the 310 bp allele, all other amplification products that were seen in 'Tirupati 3' were present. 'JL24' is highly susceptible to Kalahasti malady (Naidu 1996). The varieties, 'Narayani', 'Kadiri 6' and 'Tirupati 4' are selections from the cross, 'JL24'×'Ah316/s'. In 'Narayani', only one allele at 250 bp was seen while in 'Tirupati 4', while nine alleles were observed but none of them were similar with alleles observed in resistant genotypes, 'Tirupati 3' and 'Kalahasti'. 'Prasuna', a moderately resistant genotype, showed amplifica-

tion products of size 275, 260, 310, 375, 475 and 500 bp and some products not amplified in resistant/susceptible genotypes i.e., 525, 550, 600, 640, 700 and 800 bp. In total, 11 alleles were amplified in 'Prasuna' with pPGS seq14H6 primer. One of the resistant genotypes, 'ICGV (FDRS) 79' had alleles of size 225, 275, 330, 475, 500 and 650 bp as in var. 'Kalahasti'.

As SSR makers are co-dominant in nature, true hybrid plants can be identified through an amplification profile in hybrids. True hybrid plants should possess alleles from both parents i.e., heterozygous condition. In the F_1 of 'Narayani'×'Tirupati3', a hybrid pattern, i.e., alleles from susceptible and resistant genotypes, 'Narayani' and 'Tirupati3', were observed. F_1 was resistant to Kalahasti malady. F_1s of other crosses involving susceptible and resistant genotypes, 'Tirupati 4'×'Tirupati 3' and 'Kadiri 6'×'Tirupati 3', also exhibited heterozygosity with respect to the markers. In F_1s of 'Prasuna'×'Tirupati 3', 'Kalahasti'×'Tirupati 3' and 'Tirupati 3'×'ICG (FDRS)79', heterozygousity was clearly observed confirming hybridity; moreover, one or two alleles not seen in either of the genotypes involved were also observed in F_1s.

With 16G8 primer pair, four alleles were amplified in the material studied. Among the alleles, the allele 210 bp in size was associated with resistance to Kalahasti malady. In susceptible genotypes, there was either no amplification or alleles of size 190, 230, and 240 bp were observed. In all the F_1s, the allele 210 bp in size was amplified. In 'Tirupati 4'×'Tirupati 3', a hybrid pattern as was found in both parents i.e., alleles of size, 210 and 230 bp, was recorded.

Validation of pPGS seq14H6 and pPGS seq16G8 markers in other genotypes

In addition to screening parents and F_1s, these two primer pairs were used for further validation of 25 other genotypes whose reaction to Kalahasti malady is known. In moderately resistant genotypes i.e., 'TLG 45', 'TCGS 876', 'TCGS 584', 'Tirupati 2', 'ICGV 06138', either all or some of the alleles found to be associated with resistance i.e., 225, 260, 275, 310, 330 bp, were present (**Table 1, 2**). In amplification products through pPGS seq14H6 in susceptible genotypes, alleles not found associated with resistance i.e., 240, 250, 300, 350 bp and alleles of still higher molecular weight were recorded. Through amplification with the 16G8 primer pair, in susceptible genotypes, either there was no amplification as in 'Narayani', 'Tirupati 4', 'Kadiri 6', 'Abhaya', 'Tirupati 1', 'ICGV 91114', 'ICGV 93468', 'JL 24', 'K 134', 'K 1375', 'TCGS 821', 'ICGV 00350', 'ICGV 07022', 'ICGV 07217', 'ICGV 06039', 'ICGV 87846', 'TCGS 1043', 'ICGV 03325', and 'ICGV 08021' or amplification products of size 190, 230 or 240 bp were observed as in 'ICGV 99029', 'JL 220', 'Rohini' and 'GPBD4'. In resistant and moderately resistant genotypes an allele of 210 bp i.e., 'Tirupati 3', 'Kalahasti', 'TLG 45', 'TCGS 876', 'ICG (FDRS) 79', 'TCGS 584', 'Tirupati 2', 'ICGV 06138', was observed. In F_1s, except in the cross combination, 'Tirupati 4'×'Tirupati 3', an allele of 210 bp in size was detected. In the 'Tirupati 4'×'Tirupati 3' combination, two alleles 210 and 230 bp in size were amplified.

Screening segregating populations is very difficult as maintenance of a sick plot at the experimental farm is not possible. Hence, the mode of inheritance has not been deciphered so far. In 2002-2003 *rabi*, there was a moderate incidence of Kalahasti malady in one of the fields of RARS, Tirupati. In the cross, 'Kalahasti'×'TIR45', the F_2 segregation was a good fit to a phenotypic ratio of 13 moderately resistant: 3 resistant, a typical ratio indicating the involvement of inhibitory factors, duplicate - dominance kind of interaction (pers. obs,). In the present study, F_1 was resistant indicating that resistance to Kalahasti malady is dominant.

In the present study, alleles of size 225, 260, 275, 310 and 330 bp were amplified by pPGS seq14H6 primer only in genotypes with resistance i.e., 'Tirupati 3', 'Kalahasti' and moderately resistant genotypes, 'TLG 45', 'TCGS 876',

Table 4 Sequences of SSR primers used for screening.

Primer name	Sequence (5' - 3')
pPGSseq15C10-F	ATTCCCATGTCGTCAAGACC
pPGSseq15C10-R	GCGACGGTATTGGCTTTTAG
pPGPseq3A8-F	ATACGTGACTTGGGCCAGAC
pPGPseq3A8-R	AGTGAAAAATACACCCAACGAA
pPGPseq4G2-F	TCAACTTTGGCTGCTTCCTT
pPGPseq4G2-R	TCAACCGTTTTTCACTTCCA
pPGPseq3D9-F	TTCACCCGTACAAACCAGTG
pPGPseq3D9-R	CCTCGGCAGATCTGGAGTAA
pPGSseq13A7-F	AATCCGACGCAATGATAAAAA
pPGSseq13A7-R	TCCCCTTATTGTTCCAGCAG
pPGPseq1B9-F	CGTTCTTTGCCGTTGATTCT
pPGPseq1B9-R	AGCACGCTCGTTCTCACATT
pPGSseq12F7-F	TGTCGTTGTAAGACCTCGGA
pPGSseq12F7-R	TTGGTTTCCTTAAGGCTTCG
pPGSseq16C6-F	TTGCTACTAAGCCGAAAATGAAG
pPGSseq16C6-R	CTTGAAATTAACACATATGCACACA
pPGSseq18C5-F	GGACAGCCGGATGCTATTTA
pPGSseq18C5-R	ACATGAGTCCCTTTTCCCTT
pPGPseq2B10-F	AATGCATGAGCTTCCATCAA
pPGPseq2B10-R	AACCCCATCTTAAAATCTTACCAA
pPGSseq13A10-F	AACTCGCTTGTACCGGCTAA
pPGSseq13A10-R	AGGAATAATAACAATACCAACAGCA
pPGSseq16G8-F	CTCAAAAAGCGCTTAGCCAC
pPGSseq16G8-R	CTGCCTACTGCCTACTGCCT
pPGSseq17F6-F	CGTCGGATTTATCTGCCAGT
pPGSseq17F6-R	AGTAGGGGCAAGGGTTGATG
pPGPseq7G2-F	ACTCCCGATGCACTTGAAAT
pPGPseq7G2-R	AACCTCTGTGCACTGTCCCT
pPGPseq3A1-F	ATCATTGTGCTGAGGGAAGG
pPGPseq3A1-R	CACCATTTTTCTTTTTCACCG
pPGSseq10D4-F	ATCCCTGATTAGTGCAACGC
pPGSseq10D4-R	CGTAGGTGGTTTTAGGAGGG
pPGPseq8D9-F	TGAGTTTCCCCAAAAGGAGA
pPGPseq8D9-R	CAACAACAATACGGCCAACA
pPGPseq4H11-F	ATCACCATCAGAACGATCCC
pPGPseq4H11-R	TTTGTAGCCTTCTGGCGAGT
pPGSseq2F5-F	TGACCAAAGTGATGAAGGGA
pPGSseq2F5-R	AAGTTGTTTGTACATCTGTCATCG
pPGSseq14H6-F	GCAACTAGGGTGTATGCCGT
pPGSseq14H6-R	CAACCCTATACACCGAGGGA
pPGPseq2D12B-F	AAGCTGAACGAACTCAAGGC
pPGPseq2D12B-R	TGCAATGGGTACAATGCTAGA
pPGPseq5D5-F	AAAAGAAAGACCTTCCCCGA
pPGPseq5D5-R	GCAGGTAATCTGCCGTGATT
pPGPseq16F1-F	TGGAGGGAAAAACATTTTGG
pPGPseq16F1-R	CCTGGAGGGGTGAGAGGT
IPAHM103-F	GCATTCACCACCATAGTCCA
IPAHM103-R	TCCTCTGACTTTCCTCCATCA
IPAHM105-F	CAGAGTTTGGGAATTGATGCT
IPAHM105-R	GCCAGATCTGAGCAAGAACC
IPAHM165-F	CAACACGTTCGCTTCCAGAT
IPAHM165-R	TCACTCTCATTTCCGCCATT
IPAHM689-F	GATGACAATAGCGACGAGCA
IPAHM689-R	GTAAGCCTGCAGCAACAACA
AH041-F	CGCCACAAGATTAACAAGCACC
AH041-R	GCTGGGATCATTGTAGGGAAGG
AH193-F	CTTGCTGAAGGCAACTCCTACG
AH193-R	TCGGTTTGTCTCTTTGGTCAGTC
AH229-F	GCAAACATCTTCCTTCCCAACA
AH229-R	ATTGACGTAAGCTGCCAAGAGG
AH522-F	GTCAATGCCGAACCTCAACGTA
AH522-R	TTCACCATCATCTCCAACGCTT
PM3-F	GAAAGAAATTATACACTCCAATTATGC
PM3-R	CGGCATGACAGCTCTATGTT
PM36-F	ACTCGCCATAGCCAACAAAC
PM36-R	CATTCCCACAACTCCCACAT
PM45-F	TGAGTTGTGACGGCTTGTGT
PM45-R	GATGCATGTTTAGCACACTTGA
PM375-F	CGGCAACAGTTTTGATGGTT
PM375-R	GAAAAATATGCCGCCGTTG

'Tirupati 2', 'Prasuna', etc. In susceptible genotypes, alleles of different sizes i.e., 240 and 250 bp were amplified with this primer pair. As distinctly different alleles were amplified in resistant and susceptible genotypes, this primer seems to be useful to distinguish resistant and susceptible genotypes. This would help in marker-assisted breeding in the development of high-yielding genotypes with resistance to Kalahasti malady as the markers would be helpful in screening segregating/advanced generations of crosses for Kalahasti malady resistance in an experimental farm at the seedling stage. If markers are not available, segregating / advanced generations of crosses made to select for resistance to Kalahasti malady have to be grown in hot spot areas in farmers' fields to identify resistant plants or progenies. Due to variation in climatic and soil conditions it would be difficult to precisely identify resistant plants or progenies. There is scope for selection of susceptible plants as resistant as the population build-up of nematodes may not be that uniform at different places, even in hot spot locations. The creation of a sick plot at an experimental farm is not safe as the transmission of nematodes is through infested soil and it is also very difficult to maintain a uniform load of nematodes throughout the plot.

Another SSR primer pair, pPGS seq16G8 amplification pattern in resistant and susceptible genotypes was also distinct. Only alleles of size 210 bp were found in resistant and moderately resistant genotypes while alleles of size 190 and 230 bp were found in susceptible genotypes. In F_1s of crosses involving resistant and susceptible genotypes, alleles found in both resistant and susceptible genotypes were found and hybridity could be clearly established through the observed profile.

From the results, it is obvious that the pattern observed in resistant and susceptible genotypes has a clear association with resistance and susceptibility respectively with the two primer pairs, pPGS seq14H6 and pPGS seq16G8. The heterozygous pattern of the markers in F_1s and the resistance reaction of F_1s to Kalahasti malady further confirmed the association. However, co-segregation analysis of the markers and phenotypic data i.e. reaction to Kalahasti malady has to be further studied in mapping populations i.e. F_2 populations and recombinant inbred lines which are being developed. On further validation and confirmation of association in mapping populations, these markers would be very useful for routine screening for Kalahasti malady resistance.

As groundnut is an important oilseed crop with high food value, its area under well-managed *rabi*-summer situation is likely to increase in future. Kalahasti malady would also become a major problem in this situation. As chemical control is not cost-effective, only the development of resistant varieties offers the best solution to the problem. In this context, identification of molecular makers associated with resistance would greatly aid as robust screening tools in the development of high yielding Kalahasti malady resistant varieties with acceptable quality attributes.

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